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**13. ABSTRACT** ***(Maximum 200 Words)***

Survival of genotoxic stress is essential to the survival of any organism. The DNA damage checkpoint (DDC) is a regulatory system that controls the cellular response to DNA damage. Dysfunction of components of the mammalian DDC, such as ATM, hChk2, p53, and BRCA1, correlates with increased cancer risk. DDC mechanisms are conserved; in *Saccharomyces cerevisiae*, *ATM*-family kinase *MEC1* is required for the DDC, as are hChk2-homolog Rad53 and BRCA1-like Rad9. The powerful genetic and biochemical techniques available in the *S. cerevisiae* system present it as an ideal model organism in which to study the conserved DDC mechanisms.

Mec1-dependent phosphorylation of Rad53 correlates with the propagation of the DDC signal. Mec1 is also required for the DDC-dependent phosphorylation of Rad9, and Rad53 interacts with phosphorylated Rad9, suggesting that Rad9 acts as an adaptor for the DDC signaling pathway. A goal of this work is to characterize physical and catalytic interactions between Mec1, Rad53, and Rad9. As reported herein, I determined the contribution of Mec1 consensus phosphorylation sites within Rad9 to the functions of Rad9 in the regulation of Rad53 and the DNA damage checkpoint pathway. Further, I demonstrated the direct binding of Rad53 FHA domains to phosphorylated Rad9 peptide *in vitro*.

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## Table of Contents

## Introduction

Survival of genotoxic shock is essential to the survival of any organism. The DNA damage checkpoint (DDC) controls the cellular response to DNA damage (10, 19, 23, 24). Dysfunction of components of the mammalian DDC, such as ATM, hChk2, p53, and BRCA1, correlates with accelerated tumorigenesis, increased cancer risk, and tumor chemotherapeutic resistance (2, 8, 11, 18, 20, 21). Eukaryotic DDC mechanisms are conserved; in *Saccharomyces cerevisiae*, *ATM*-family kinase *MEC1* is required for the DDC, as are Rad53 and Rad9 (1, 5, 12, 14, 16, 19, 22, 25, 29, 30, 31). Rad53 is the founding member of a kinase family implicated in DDCs, including mammalian homolog hChk2 (3, 4, 12, 13, 15, 30). Rad9 shares homology with the BRCA1 C-terminus (32). *S. cerevisiae* thus provides a powerful genetic system in which to study the conserved DDC mechanisms.

The Mec1-dependent phosphorylation of Rad53 correlates with the propagation of the DDC signal (17, 26, 27). Mec1 is also required for the DDC-dependent phosphorylation of Rad9 (26, 7, 28), leading to the binding of Rad53 to phospho-Rad9 via the FHA domains of Rad53 (26, 6). The two objectives of this project are: to define the molecular and catalytic interaction between Mec1, Rad53, and Rad9 that lead to the activation of Rad53 by the DDC; and to identify and begin characterization of mammalian homologs of *RAD53*. As I reported last year, the second objective was completed and published by multiple independent laboratories within the first year of my project. In the past year, I focused my time on detailed experiments within the later two tasks of my first objective, namely a detailed analysis of the interaction of Rad53 with Mec1 phosphorylation sites within Rad9.

## Progress on Objective 1: Characterization of physical and catalytic interactions of Mec1

### *Task 3: characterization of physical interactions between Mec1, Rad53, and Rad9*

To characterize the formation of a complex including Mec1, Rad53, and Rad9, I sought to identify by immunoprecipitation a complex containing Mec1 and Rad9 or Rad53. Despite repeated attempts by myself and other members of the laboratory, we have been unable to coimmunoprecipitate such a complex. Genetic and biochemical data, however, indicates that Rad9 is likely a direct substrate of Mec1 *in vivo*, and, as I reported from my studies last year, Mec1 phosphorylates an ATM-family consensus [S/T]Q site within Rad9 *in vitro*. To expand our understanding of the Rad53-Rad9 interaction first discovered in our laboratory, I studied the possibility that the putative Mec1 phosphorylation [S/T]Q sites within Rad9 are *bona fide* DNA damage-induced phosphorylation sites that serve as Rad53 FHA domain docking sites *in vivo*. This is especially exiting as, though the FHA domain is a novel phosphorylation-dependent protein binding domain with several literature reports of *in vitro* binding specificities, not one *in vivo* FHA domain binding site has yet been described.

As I reported last year, mutagenesis of [S/T]Q sites within Rad9 determined that a [S/T]Q cluster domain (SCD) within Rad9 is required for the DDC-induced Rad9 phosphorylation and interaction of Rad9 with Rad53. The Rad9 SCD contains a total of six [S/T]Q sites. To determine if a single site has a dominant contribution to Rad9 phosphorylation and interaction with Rad53, I tested a series of *rad9* mutants containing single alanine substitutions of individual SCD sites. Mutation of single [S/T]Q sites within the SCD had little apparent effect on the DNA damage induced phosphorylation of Rad9, immunoprecipitation of Rad9 with Rad53, or Rad53 phosphorylation (Figure 1). Since simultaneous substitution of all six sites does inactivate these functions, these data suggest that there are multiple phosphorylation sites within this group, and that Rad53 interacts redundantly with some or all of these sites.

To characterize the ability of a single Rad9 SCD site to support the interaction of Rad9 with Rad53, I examined the rescue of Rad9 and Rad53 DNA damage checkpoint regulation after restoration of single [S/T]Q residues into an otherwise mutant SCD. Reintroduction of a wildtype residue at Rad9 S435 best restored the DNA damage-induced slower mobility forms of Rad9 (Figure 2). However, reintroduction of Rad9 S435 only modestly rescued the DNA damage-induced Rad9 coimmunoprecipitation with Rad53, and the phosphorylation of Rad53 (Figure 2). By contrast, reintroduction of Rad9 T390 moderately restored the DNA damage-induced slower mobility forms of both Rad9 and Rad53 (Figure 2). However, even this add-back mutant only partially

rescued coimmunoprecipitation of Rad9 with Rad53, and no single Rad9 cluster add-back allele fully restored the checkpoint-induced phosphorylation or coimmunoprecipitation of Rad9 and Rad53 (Figure 2). These data indicate that the Rad9 [S/T]Q cluster sites may act in an additive or cooperative manner to recruit Rad53. Moreover, these results extend the absolute correlation between the ability of Rad9 to coimmunoprecipitate with Rad53 and the damage-regulated phosphorylation of Rad53, supporting the model that Rad9 functions as an adaptor for the DDC signaling pathway.

I also explored the impact of Rad9 SCD mutations on the function of the $G_2$/M arrest induced by the DDC (9). My analysis of the $G_2$/M DDC in wildtype, *rad9*$^{6xA}$, *rad9*Δ, and *rad53*$^{FHA2\text{-}NVS}$ demonstrated that *rad9*$^{6xA}$ has defects similar to those of the *rad53* cells (Figure 3). Consistent with the ability of the T390 add-back mutant to significantly recover the DNA damage-induced interaction of Rad9 with Rad53, reintroduction of Rad9 T390 to *rad9*$^{6xA}$ also partially recovered the *RAD53*-dependent $G_2$/M arrest (Figure 3). However, this rescue was weak, suggesting that the regulation of Rad53 via an intact Rad9 SCD is required for the full action of the *RAD53*-dependent $G_2$/M DNA damage checkpoint arrest. Readdition of S435 to the *rad9*$^{6xA}$ mutant only weakly recovering the coimmunoprecipitation of Rad9 with Rad53, was less able to recover the $G_2$/M arrest (Figure 3).

Survival of genotoxic stress requires the activity of the DNA checkpoint pathways, as well as the DNA repair systems. An understanding of this process is essential for the efficacious use of chemo- and radiotherapy. In *S. cerevisiae*, loss of *RAD9* impairs both the DNA damage checkpoint pathway and DNA repair, and significantly decreases the viability of cells challenged with DNA damage. Mutation of the Rad9 SCD (*rad9*$^{6xA}$) causes a very slight reduction in survival of DNA damage, which, like the *RAD9*-dependent phosphorylation of Rad53 in this mutant, is largely recovered by the restoration of a wildtype residue at T390 (Figure 4A). The additional mutation of another [S/T]Q site at T603 (*rad9*$^{7xA}$) cells causes a greater loss of viability (Figure 4A).

Tof1 was identified in a yeast two-hybrid screen with DNA topoisomerase I, and is required for the activation of Rad53 by the *RAD9*-independent DNA replication checkpoint pathway (33, 34). Inhibition of replication with hydroxyurea (HU) in DNA replication checkpoint mutants, such as *tof1Δ*, is thought to cause replication forks collapse, causing DNA damage (35, 36). Indeed, the deletion of *TOF1* causes HU-induced Rad53 phosphorylation to be *RAD9*-dependent (34). Therefore, I determined whether the *rad9*$^{7xA}$ mutation decreases the HU survival of a *tof1*Δ mutant. Wildtype, *tof1*Δ, and *rad9*$^{7xA}$ strains are similarly viable when grown on HU-containing media, whereas the *tof1*Δ *rad9*$^{7xA}$ double mutant is very sensitive to growth on HU (Figure 4B). The *tof1*Δ *rad53*Δ mutant is more sensitive to HU than the *tof1*Δ *rad9*$^{7xA}$ mutant (Figure 5B), suggesting that even if Rad53 activation is compromised, Rad53 still contributes to the survival of DNA damage. Notably, a *rad53*Δ background uncovers a more significant loss of HU survival upon *TOF1* deletion than normally observed in a wildtype background (Figure 4B).

In addition to interacting with and thereby regulating the phosphorylation of Rad53, Rad9 also regulates the phosphorylation of another DDC component, Chk1. I am currently examining whether Rad9 SCD mutants that are defective for Rad53 regulation are similarly impaired for Chk1 regulation. I am also developing a method to purify *in vivo* phosphorylated Rad9 from yeast cell lysates, in order to directly identify Rad9 phosphorylation sites by mass spectroscopy.

***Task 4: in vitro reconstruction of the Rad53-Rad9 interaction***

One of the outstanding questions about the Rad53-Rad9 interaction is whether the interaction is direct, or via another polypeptide or other intermediary. To distinguish these possibilities, I sought to demonstrate Rad53 binding to Rad9 *in vitro* using components generated independent of *S. cerevisiae*. The ability of Rad9$^{6xA+T390}$ to enable a significant proportion of the Rad9 interaction with Rad53 implies that T390 is a major functional site for Rad53 binding *in vivo*. To determine if Rad53 binds directly to Rad9 T390 in a phosphorylation-dependent manner, I measured the increase of surface plasmon resonance caused by binding of soluble, bacterially produced Rad53 FHA domains to synthetic Rad9 T390 peptides immobilized on a BIAcore

sensor chip. Rad53 FHA1 specifically interacted with the phosphorylated Rad9 T390 peptide (*P*-T390), and mutation of two conserved FHA domain residues abolished this binding (Figure 5). Similar to FHA1, GST-Rad53 FHA2 preferentially bound the phosphorylated Rad9 T390 peptide, demonstrating an affinity lost upon mutation of conserved FHA2 residues (Figure 5). On average, Rad53 FHA1 bound the phosphorylated Rad9 T390 peptide with a $K_D$ of 2.5 μM (*s*=0.3, n=6), and GST-Rad53 FHA2 bound with a $K_D$ of 1.4 μM (*s*=0.3, n=3), though the observed affinity of GST-FHA2 may have been artificially increased due to the ability of GST to homodimerize. Taken together, these results show that the Rad53 FHA domains can directly and specifically bind Rad9 peptides phosphorylated at ATM-family consensus [S/T]Q phosphorylation sites.

## Key Research Accomplishments

- Characterized the contribution of the Rad9 SCD to
  - Rad9 phosphorylation
  - Interaction of Rad9 with Rad53
  - Rad53 phosphorylation
  - Function of the G2/M DNA damage checkpoint arrest
  - Survival of genotoxic stress
- Demonstrated *in vitro* the direct binding of Rad53 FHA domains to a phosphorylated Rad9 SCD peptide

## Reportable Outcomes

1. Manuscripts, abstracts, and presentations
2. Patents and licenses applied for and/or issued
3. Degrees obtained
4. Development of cell lines, tissue or serum repositories
5. Informatics
6. Funding applied for
7. Employment or research opportunities applied for

## Summary

Significant progress has been made in meeting the goals of the first objective of this proposal. I identified a cluster of putative Mec1 phosphorylation sites within Rad9 that are required for the phosphorylation of Rad9, the interaction of Rad53 with Rad9, and the survival of genotoxic stress. I determined the contribution of individual sites within this cluster to the function of Rad9 in regulating Rad53 and the G2/M DNA damage checkpoint. I demonstrated that both Rad53 FHA domains bind a Rad9 SCD site *in vitro* when phosphorylated. I am exploring whether the Rad9 SCD contributes to Rad9 activities other than regulation of Rad53, and am seeking direct biochemical evidence that sites within the Rad9 SCD are phosphorylated in response to DNA damage. I am preparing a manuscript describing these results, and will be submitting it to a leading journal shortly.

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## Figures

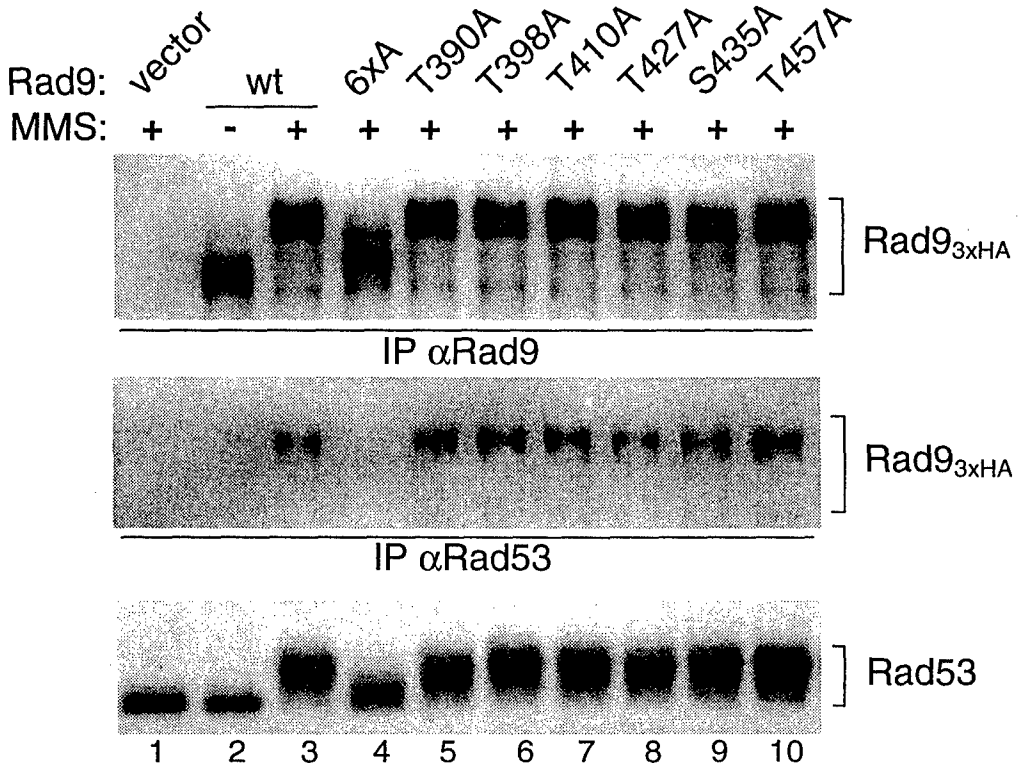


**Figure 1:** Mutagenesis of the Rad9 SCD: 1. Single site mutations. Wildtype Rad9 (wt), an all-alanine SCD mutant (6xA), and single alanine substitutions of SCD sites were immunoprecipitated anti-Rad9 (top) or anti-Rad53 (middle) and blotted anti-HA (top and middle). Corrsponding lysates (50μg) were blotted anti-Rad53 (bottom).

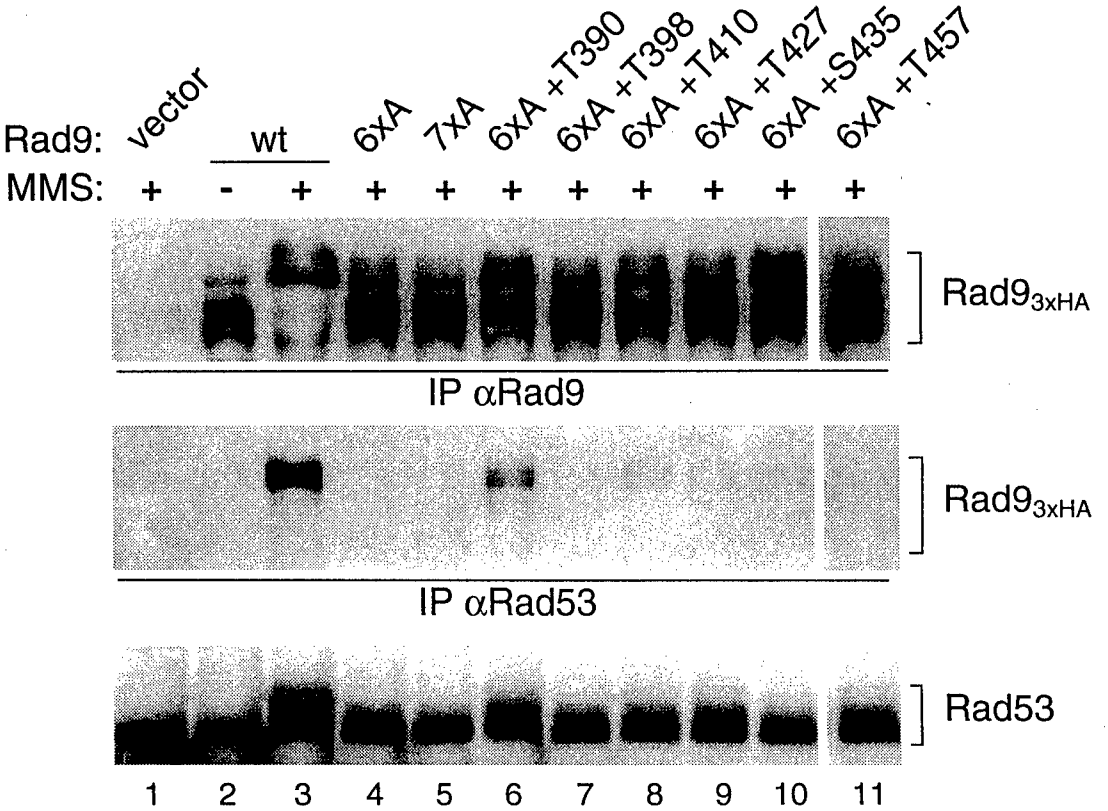


**Figure 2:** Mutagenesis of the Rad9 SCD: 2. Single wildtype sites in a multiple-alanine mutant SCD. Wildtype Rad9 (wt), all-alanine SCD mutants (6xA and 7xA), and multiple alanine SCD mutants with a single wildtype residue at the indicated site were immunoprecipitated anti-Rad9 (top) or anti-Rad53 (middle) and blotted with anti-HA (top and middle). Corresponding lysates (50μg) were blotted anti-Rad53 (bottom).

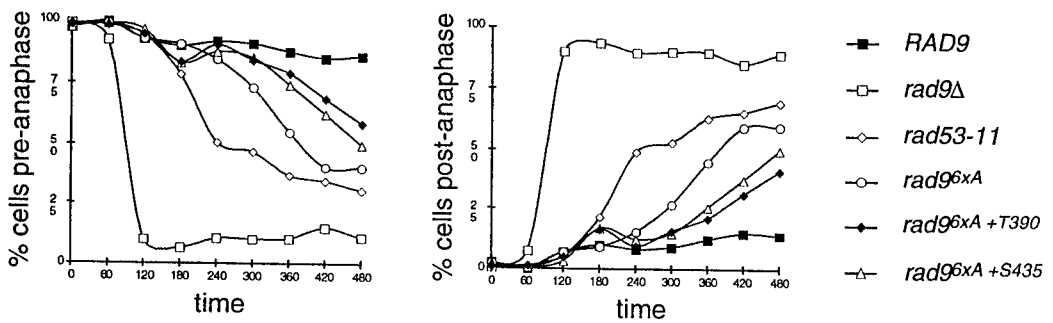


**Figure 3:** Mutation of the Rad9 SCD impairs the DNA damage checkpoint arrest at $G_2$/M. The ability of Rad9 SCD mutations to arrest as pre-anaphase cells at the $G_2$/M checkpoint arrest in response to DNA damage was compared to the known mutations in the DDC.

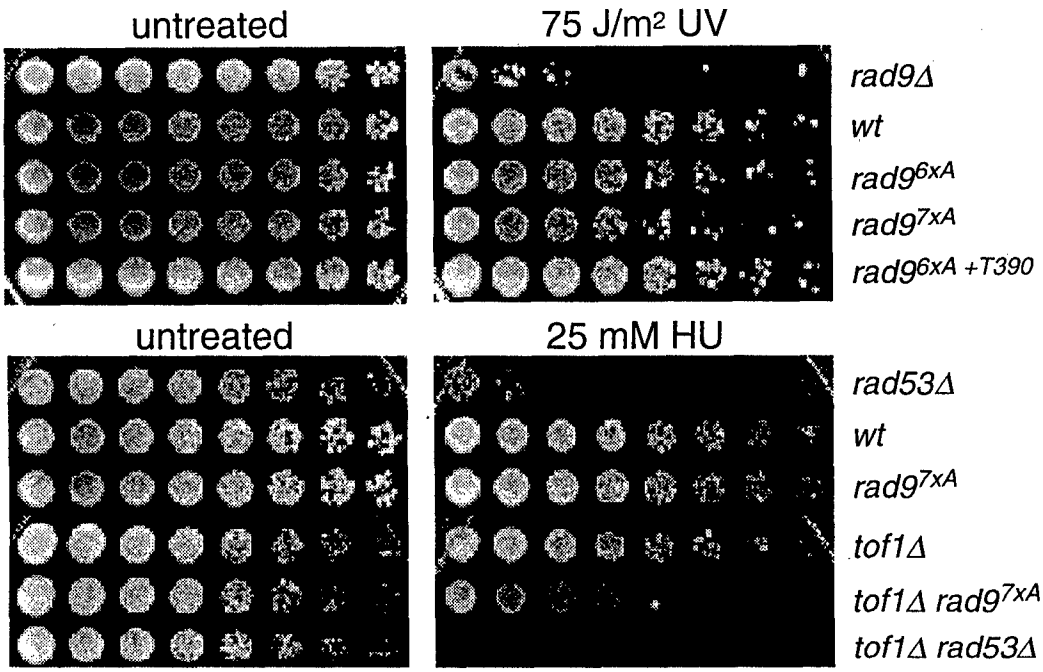


**Figure 4:** Rad9 SCD mutations impair the survival of DNA damage. Mutation of [S/T]Q sutes within Rad9 reduce the viability of cells exposed to UV irradiation (top) and *tof1*Δ cells exposed to HU (bottom).

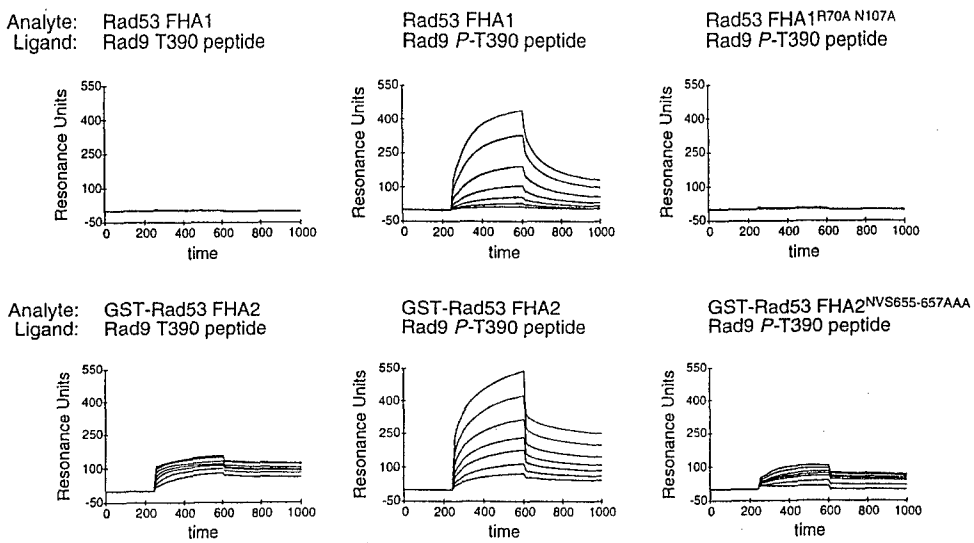


**Figure 5:** Surface plasmon resonance measurements of Rad53 FHA domains binding a Rad9 SCD peptide. Bacterially produced Rad53 FHA domain fusion proteins were flowed over the surface of a BIAcore sensor chip coated with the indicated Rad9 SCD site T390 peptide. Binding of the fusion proteins to the surface of the sensor chip is indicated by an increase of the surface plasmon resonance.

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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